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CD147 monoclonal antibodies induce homotypic cell aggregation of monocytic cell line U937 via LFA-1/ICAM-1 pathway

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SUMMARY

CD147 is a 50 000–60 000 MW glycoprotein of the immunoglobulin superfamily broadly expressed on haemopoietic cell lines and peripheral blood cells. In the present study, six monoclonal antibodies (mAbs) directed against the CD147 protein were generated. The antigen defined by the generated CD147 mAbs is widely expressed on haemopoietic cell lines, peripheral blood cells and is a lymphocyte activation-associated cell surface molecule. The generated CD147 mAbs precipitated a broad protein band from U937 cells of 45 000–65 000 MW under reducing conditions. Functional analysis indicated that the CD147 mAbs markedly induced homotypic cell aggregation of U937 cells, but not K562 cells. The CD147 mAb-induced cell aggregation was inhibited by leucocyte function-antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) mAbs. However, the expression of LFA-1 and ICAM-1 molecules on U937 was not altered by CD147 mAb treatment. The U937 cell aggregation induced by CD147 mAb was also inhibited by ethylenediamine tetra-acetic acid (EDTA), sodium azide and when incubated at 4°. We therefore propose that the binding of CD147 mAb to CD147 molecule, which mimics the natural ligand binding, may generate intracellular signals that activate LFA-1/ICAM-1 intercellular adhesion pathway.

INTRODUCTION

The CD147 molecule is a leucocyte surface protein with a molecular mass of 50 000–60 000 MW which has recently been clustered at the 6th International Workshops on Human Leukocyte Differentiation Antigen.[1] Other names for this molecule include human basigin, M6, and extracellular matrix metalloproteinase inducer (EMMPRIN).[1–4] Cloning and sequence analysis of the encoding cDNA indicate that it is a member of the immunoglobulin superfamily and is the species homologue of rat OX-47 antigen, mouse basigin or gp42 and chicken HT7 molecules.[2,3] Recently, the rabbit homologue was described.[5] The putative transmembrane region of the CD147 molecule is strongly conserved between human and its species homologues.[1–3] Interestingly, the hydrophobic stretch present in the transmembrane regions of CD147 protein is interrupted by a charge residue, a glutamic acid, and contains a leucine-zipper motif.[3] Charge residue and leucine-zipper in the transmembrane are potential protein–protein interaction motifs.[6–9]

At the 6th workshop, five mAbs were assigned as CD147 monoclonal antibodies (mAbs).[1] Cellular expression analysis using workshop mAbs indicated that CD147 is broadly expressed on haemopoietic and non-haemopoietic cell lines.[1,3,10] Within peripheral blood cells, CD147 is expressed on all leucocytes, red blood cells, platelets and endothelial cells.[1,3,10] Some CD147 mAbs inhibited homotypic aggregation of oestrogen-dependent breast cancer cell line MCF-7, as well as MCF-7 cell adhesion to type IV collagen, fibronectin and laminin.[1] Furthermore, a soluble recombinant CD147 form was produced by fusing the cDNA coding for the entire extracellular domain of the CD147 molecule to DNA encoding for constant domains of human immunoglobulin-1 (IgG1). The soluble recombinant CD147 molecules could bind to endothelial cells and fibroblasts.[1] Together, all these functional analysis data suggest that the CD147 molecule is a potential adhesion molecule.[1]

In the present study, six mAbs specific for CD147 molecule were raised and their characteristics were studied. Functional analysis demonstrated that the generated CD147 mAbs induced homotypic cell aggregation of U937. The U937 cell aggregation induced by CD147 mAb was found to be leucocyte function-antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1)-dependent pathway.

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MATERIALS AND METHODS

Cells, cell activation and cell lines

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood from healthy donors

by density gradient centrifugation over Ficoll–Hypaque solution (Sigma, St. Louis, MO). PBMC were washed three times with RPMI-1640 medium (Gibco, Grand Island, NY) and used for cell activation and immunophenotyping. For cell activation, PBMC were cultured at 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 40 μg/ml gentamicin and 2·5 μg/ml amphotericin B in the presence of 2 μg/ml phytohaemagglutinin (PHA; Murex, Dartford, UK) for 1–5 days in a humidified atmosphere with 5%CO_2 at 37°.

Human T-cell lines (Jurkat, Molt4 and Sup T1), a B-cell line (Daudi), a monocytic cell line (U937), and an erythroid/myeloid cell line (K562) were used in this study. All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS (except Sup T1 with 15% FBS) and antibiotics in a 5%CO_2 incubator. COS7 cells were maintained in minimum essential medium (MEM; Gibco) containing 10% FBS and antibiotics at 37° in a 5%CO_2 atmosphere.

Plasmid DNA and antibodies

The CDM8-derived expression plasmid H34, containing the cDNA encoding M6 (CD147) antigen,[3] and H04, encoding the CD1a antigen (W. Kasinrerk, unpublished observation) were transformed into *Escherichia coli* MC1061/p3. Plasmid DNA encoding CD147 and CD1a proteins were isolated from transformed *E. coli* by alkaline lysis and purified using ethidium bromide caesium chloride ultracentrifugation. The isolated plasmid DNA were proved for expression of the encoded proteins by using the COS cell expression system and indirect immunofluorescent staining of the transfected COS cells with specific mAbs.

The mAbs MT4 (IgM isotype) and MT8 (IgM) specific for CD4 and CD8 molecules, respectively (W. Kasinrerk, unpublished observations), as well as irrelevant mAbs, P-3E10 (IgG2a) and P-3C7 (IgG2a) were generated in our department. MEM-25 (IgG1; CD11a),[11,12] MEM-30 (IgG1; CD11a),[11,12] MEM-48 (IgG1; CD18)[12] and MEM-111 (IgG2a; CD54 or ICAM-1)[13] were kindly provided by Dr Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Praha, Czech Republic). HI111(IgG1; CD11a), ICRF44 (IgG1; CD11b), B-ly6 (IgG1; CD11c), 6·7 (IgG1; CD18), HA58 (IgG1; CD54) and 9F10 (IgG1; CD49d) are phycoerythrin (PE) conjugates (Pharmingen, San Diego, CA), were kindly provided by Becton Dickinson (Thailand).

To purify the mAbs of interest, affinity chromatography was used. IgM isotype mAbs were purified from ascites by using antimouse IgM coated sepharose column (Zymed Laboratory Inc., San Francisco, CA) according to the method described elsewhere.[14] IgG isotype mAbs were purified by using protein A coated sepharose column (Pharmacia, Uppsala, Sweden)

Hybridoma production

Female BALB/c mice were i.p. immunized three times at weekly intervals with 1×10^7 Sup T1 or Molt4 cells. After immunization, mice were i.v. boosted with 1×10^6 cells. Splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by a standard hybridoma technique using 50% polyethylene glycol.[15] After hypoxanthine, aminopterin and thymidine (HAT) medium selection, culture supernatants were firstly analysed for antibody reactivity against the immunizing cells using indirect immunofluorescence assay. The positive supernatants were then differentially screened for CD147 specificity using plasmid DNA H34 transfected- and H04 transfected-COS cells by indirect immunofluorescence assay. The positive hybridomas were subcloned for three rounds. The isotype of antibodies was determined using an isotyping enzyme-linked immunosorbent assay (ELISA) kit (Sigma). By this procedure, six CD147 mAbs, designated M6-1D4, M6-2F9, M6-1F3, M6-2G11, M6-1E9 and M6-1B9, were obtained. M6-1D4, M6-2F9, M6-1F3 and M6-2G11 were IgM isotype, whereas M6-1E9 and M6-1B9 were IgG2a and IgG3 isotypes, respectively.

Diethylaminoethyl (DEAE)–dextran transfection of COS cells

Plasmid DNA encoding human leucocyte surface antigens were transfected into COS cells using the DEAE–dextran transfection method.[3] Briefly, 1×10^6 COS cells were transfered to 6 cm tissue culture dishes (Nunc, Roskilde, Denmark) on the day before transfection. Cells were incubated with 2 ml of MEM containing 250 μg/ml DEAE–dextran (Sigma), 400 mM chloroquine diphosphate (Sigma) and 2 μg DNA for 3 hr at 37°. Supernatant was removed and cells were treated with 10% dimethylsulphoxide (DMSO) in phosphate-buffered saline (PBS) for 2 min at room temperature. Cells were then cultured in MEM containing 10% FBS overnight, washed once, and recultured with the same medium for another 2 days to allow expression of the corresponding proteins.

Immunofluorescence analysis

Cells were analysed by indirect immunofluorescence using FITC-conjugated sheep $F(ab')_2$ antimouse immunoglobulin antibodies (Immunotech/Coulter Corporation, Miami, FL). To block non-specific FcR-mediated binding of mAb, cells were preincubated for 30 min at 4° with 10% human AB serum before staining. Membrane fluorescence was analysed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer. Individual populations of blood cells were gated according to their forward and side scatter characteristics.

Labelling of cells and immunoprecipitation

For surface labelling, washed cells in PBS were biotinylated with Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) (5 mM) for 1 hr at 4° and reactions quenched by washing once with 1 mM glycine in PBS and then twice with PBS. Cells (1×10^7) were solubilized in 1 ml lysis buffer (1% NP-40, 50 mM Tris–HCl pH 7·4, 150 mM NaCl, 5 mM ethylenediamine tetra-acetic acid (EDTA), 5 mM iodoactamide, 2 mM phenylmethyl-sulphonyl fluoride (PMSF), and 10 μg/ml aprotinin). Cell lysates were precleared with protein A–Sepharose beads coated with non-specific mAb. Precleared lysates were then mixed with purified mAb coated protein A–Sepharose beads at 4° for 24 hr. For IgM isotype mAbs, protein A–Sepharose beads were first coated with sheep antimouse IgM (Sigma) and then with tested purified IgM mAbs. After immunoprecipitation and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), biotinylated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS for 1 hr at room temperature. The blocked membrane was incubated for 1 hr at room temperature with avidin–peroxidase (Dako, Glostrup, Denmark), and the

biotinylated proteins were visualized by the chemiluminescence detection system (Pierce).

Homotypic cell aggregation assay

U937 and K562 were washed three times with RPMI-1640 medium and resuspended to a concentration of 2×10^5 cells/ml with RPMI-1640 supplemented with 10% FBS and antibiotics. One hundred and fifty microlitres of cell suspension was then distributed into 96-well flat-bottomed microplate (Nunc). Tested mAbs were added to each well with a final concentration of 10 μg/ml to obtain the total volume of 250 μl. Plates were incubated in a CO_2 incubator and examined for homotypic cell aggregation under a phase-contrast inverted microscope (Olympus, Tokyo, Japan) at various time points. The degrees of cell aggregation were scored as follows; no aggregation (–), 1–5 cells/aggregate (1+), 6–10 cells/aggregate (2+), 10–15 cells/aggregate (3+), greater than 15 cells/aggregate (4+). Photographs were taken with an Olympus camera under an inverted microscope.

In order to study the effect of LFA-1 and ICAM-1 antibodies on cell aggregation, mAbs to CD11a, CD18 and CD54 molecules (40 μg/ml) were added into wells containing U937 and CD147 mAb. Cell aggregation was then determined and scored as described above.

Adhesion molecule expression on CD147 mAb treated U937

The expression of various adhesion molecules on M6-1D4 mAb treated U937 was examined by flow cytometry. U937 ($1{\cdot}2 \times 10^4$ cells/ml) were incubated with 10 μg/ml M6-1D4 mAb for 24 hr in a CO_2 incubator. Cells were then washed twice and stained with PE-labelled HI111(CD11a), ICRF44 (CD11b), B-ly6 (CD11c), 6.7 (CD18), HA58 (CD49d) and HA58 (CD54) mAbs by direct immunofluorescence technique.

RESULTS

Production of CD147 mAbs and their reactivity

To generate CD147 mAbs, mice were immunized with M6 expressing haemopoietic cell lines and the specific antibodies were screened by using the indirect immunofluorescence technique. Six CD147 mAbs, designated M6-1D4(IgM), M6-1F3(IgM), M6-2F9(IgM), M6-1B9(IgG3), M6-2G11 (IgM) and M6-1E9(IgG2a), which strongly bound to CD147 expressing transfected COS cells but not to mock (CD1a) transfected COS cells, were obtained (Fig. 1). The reactivity of these mAbs to various cell lines was determined by flow cytometry. All generated CD147 mAbs strongly reacted with all cell lines tested including the human monocytic cell line, U937; the erythroid/myeloid cell lines, K562; the T-cell lines, Jurkat, Sup T1, Molt4 and the B-cell line, Daudi (Table 1).

Reactivity of the CD147 mAbs to PBMC was determined. All generated CD147 mAbs showed positive reactivity to monocytes ($n=7$) (Fig. 2a). In lymphocytes ($n=7$), according to the staining profiles, the generated CD147 mAbs were separated into three groups. M6-1D4 and M6-1E9 showed positive reactivity, M6-1F3 and M6-1B9 showed weak reactivity, while M6-2F9 and M6-2G11 reactivity was not detected (Fig. 2b). Within red blood cells (RBC) ($n=4$), only two mAbs, M6-1D4 and M6-1E9, showed positive reaction (Fig. 2f). The effect of neuraminidase treatment on binding of CD147 mAbs was studied. Neuraminidase treatment of RBC ($n=3$) and PBMC ($n=3$) had no effect on the binding of all CD147 mAbs (data not shown).

Reactivity of CD147 mAbs with PHA-activated lymphocytes was also analysed ($n=4$). As shown in Fig. 2(c–e), the reactivity of all CD147 mAbs was slightly increased on lymphocytes stimulated with PHA for 1 day (Fig. 2c) when compared to the staining profile of freshly isolated lymphocytes (Fig. 2b). However, after 3 days of PHA activation,

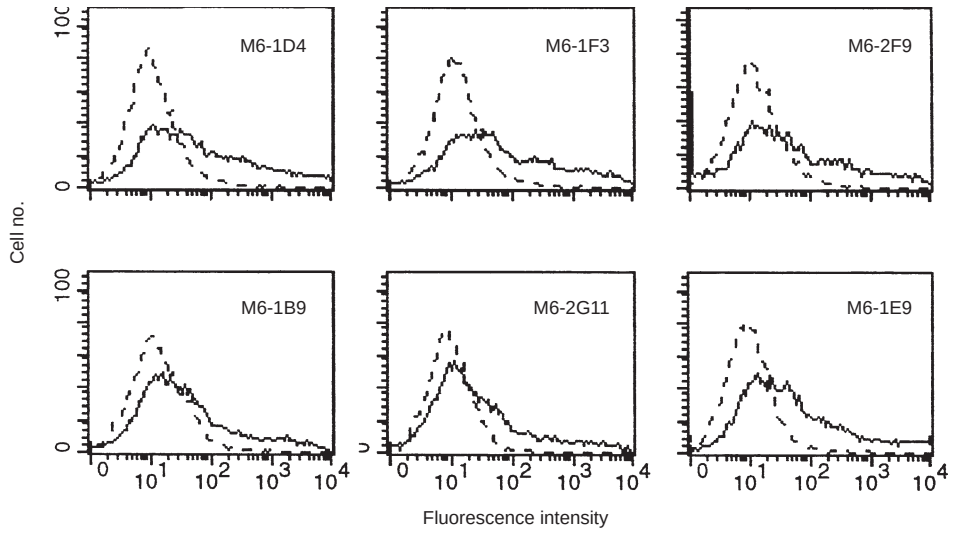

Figure 1. Immunofluorescence analysis of the reactivity of CD147 mAbs with CD147 expressing transfected COS cells. Solid lines represent the immunofluorescence profiles of CD147 (H34 DNA) transfected COS cells stained with the indicated mAbs and dashed lines represent background fluorescence of CD1a (H04 DNA) transfected COS cells stained with the indicated mAbs.

Table 1. Reactivity of CD147 mAbs with haemopoietic cell lines

	Relative fluorescence index (RFI)* with antibody					
Cell line	M6-1D4	M6-1F3	M6-2F9	M6-1B9	M6-2G11	M6-1E9
U937	145	97	108	47	14	81
K562	60	51	55	15	11	30
Jurkat	45	22	30	14	10	24
Sup T1	121	26	54	70	58	137
Molt4	50	44	46	17	14	32
Daudi	79	67	60	60	69	168

*Relative fluorescence index (RFI) is the ratio of mean fluorescence intensity of test antibody versus mean fluorescence intensity of isotype matched control.

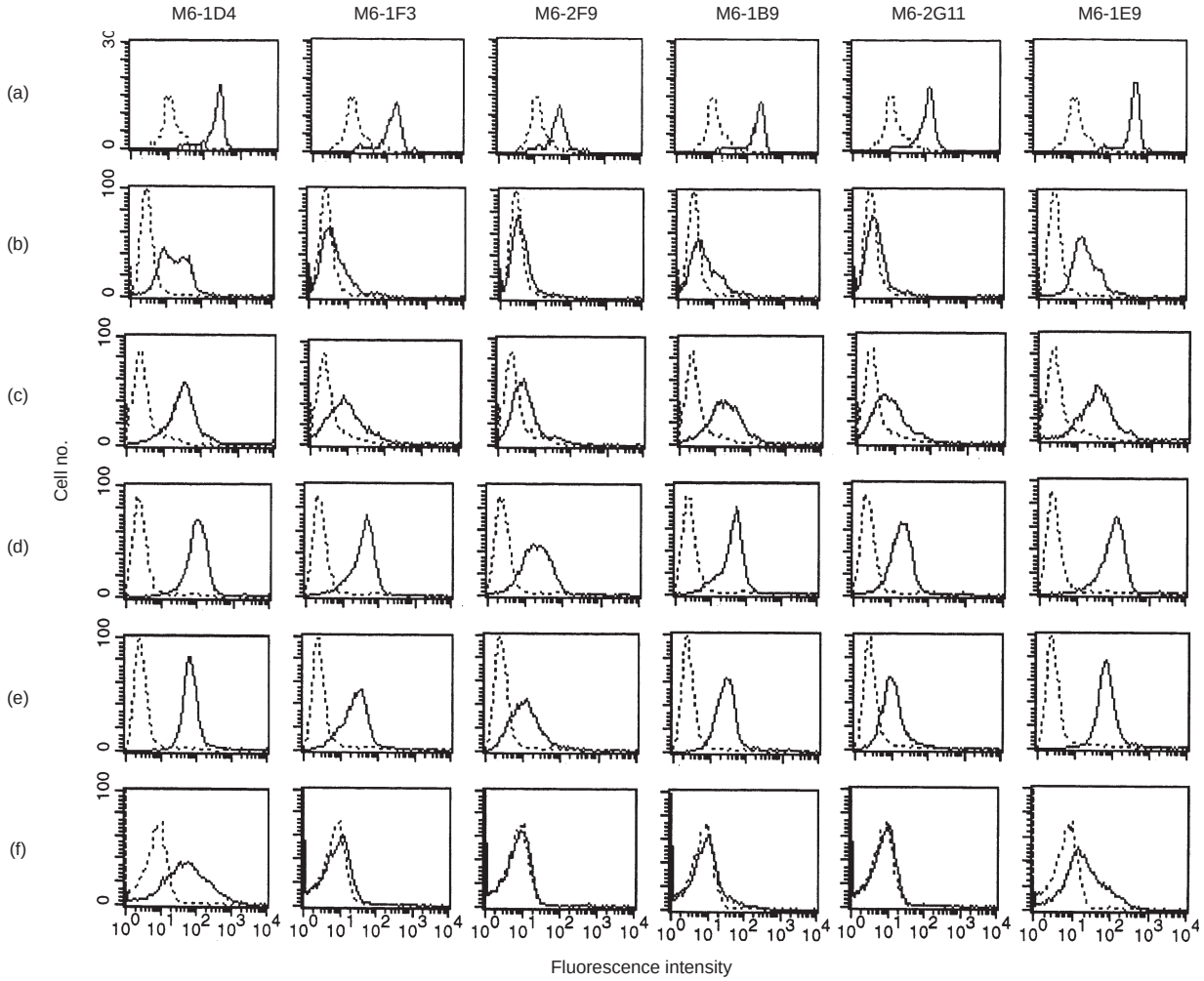

Figure 2. Immunofluorescence analysis of the reactivity of CD147 mAbs with monocytes (a), freshly isolated lymphocytes (b), 1 day PHA activated lymphocytes (c), 3 days PHA activated lymphocytes (d), 5 days PHA activated lymphocytes (e) and RBC (f). Solid lines represent the immunofluorescence profiles of cells stained with indicated mAbs and dashed line represent background fluorescence of negative control mAb.

lymphocytes became clearly positive with all CD147 mAbs (Fig. 2d). After 5 days of PHA activation, the degree of positive was almost the same as those observed after 3 days activation (Fig. 2d,e). In the same condition, expression of CD25 molecules was strongly up-regulated by PHA activation (data not shown).

Biochemical characterization of cell surface antigens recognized by CD147 mAbs

To analyse the molecular weight of the recognized molecules, we performed immunoprecipitation of lysates of surface-biotinylated U937 with purified CD147 mAbs (M6-1D4,

M6-1F3, M6-2F9, M6-1E9) and isotype-matched control mAbs (MT8 and P-3C7). The immunoprecipitation experiments using CD147 mAbs revealed a very broad protein band of ≈45 000–65 000 MW under reducing conditions (Fig. 3). The isotype-matched control mAbs, as expected, did not precipitate any protein from the U937 lysates (Fig. 3). The broad precipitated protein band seem to be composed of multiple protein bands which is obviously observed in Fig. 3(a) lanes 2 and 3. Immunoprecipitation of lysates of surface-biotinylated Molt4 with purified M6-1D4 was also performed. The similar multiple protein bands range from 45 000–65 000 MW were also obtained (data not shown).

CD147 mAbs induce homotypic cell aggregation of monocytic cell line U937

To study the function of CD147 molecule, mAbs M6-1D4, M6-1F3, M6-2F9 and M6-1E9 were purified from ascites using affinity chromatography. U937 and K562 were cultured in the presence or absence of purified CD147 mAbs or isotype-matched control mAbs. Cell aggregation was observed under inverted microscope. As shown in Table 2 and Fig. 4, M6-1D4 strongly induced U937 homotypic cell aggregation while M6-1F3 and M6-2F9 induced a lower degree of aggregation. M6-1E9, in contrast, did not induce cell aggregation. MT4, MT8 and P-3E10 mAbs were used as isotype-matched controls. These isotype-matched control mAbs did not induce U937 aggregation. All CD147 mAbs, however, did not induce K562 cell aggregation (Table 2).

Time kinetics of M6-1D4 in the induction of cell aggregation was studied. As shown in Table 3, induction of U937 aggregation could not be observed when cells were cultured with M6-1D4 for 2, 4, and 8 hr. Cell aggregation was occurred about 18 hr after administration of CD147 mAb and reached the maximal aggregation (4+) after 24 hr culturing. The aggregation was sustained for up to 4 days after culturing in the presence of CD147 mAb.

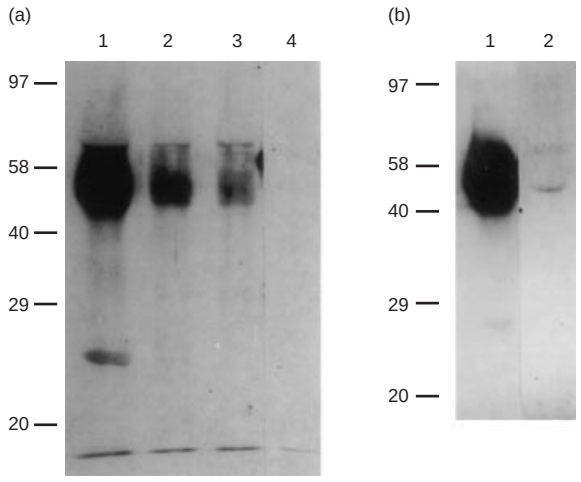

Figure 3. Biochemical characterization of the cell surface antigen recognized by CD147 mAbs. (a) SDS–PAGE analysis of immunoprecipitates obtained from lysates of biotin labelled U937 cells using M6-1D4 (lane 1), M6-1F3 (lane 2), M6-2F9 (lane 3), MT8 isotype matched control mAb (lane 4) and (b) using M6-1E9 (lane 1) and P-3C7 control mAb (lane 2). Electrophoresis was performed under reducing conditions. The positions of molecular mass markers are indicated on the left in MW ($\times 10^3$).

Table 2. Homotypic cell aggregation of U937 induced by CD147 mAbs

	Degree of cell aggregation	
Purified mAbs	U937	K562
M6-1D4	4+	–
M6-1F3	2+	–
M6-2F9	3+	–
M6-1E9	–	–
MT4	–	–
MT8	–	–
P-3E10	–	–
none	–	–

U937 or K562 cells were cultured for 24 hr in the presence or absence of mAb (10 μg/ml). Homotypic cell aggregation was determined under an inverted microscope. Degree of cell aggregation was graded as described in Materials and Methods.

Results are representative of three separate experiments.

Effect of EDTA, sodium azide and temperature on homotypic cell aggregation induced by M6-1D4

The effect of EDTA and sodium azide was investigated. In the presence of a low dose of EDTA (0·1–0·5 mM) or sodium azide (0·0125–0·05%), no effect on M6-1D4-induced cell aggregation was observed (Table 4). However, at 1 mM of EDTA and 0·1% of sodium azide, cell aggregation was inhibited (Table 4). These concentrations of EDTA and sodium azide did not affect cell viability (by trypan blue dye-exclusion test) after 24 hr culturing. The effect of temperature on M6-1D4 induced cell aggregation was also studied. We found that homotypic cell aggregation induced by M6-1D4 was temperature dependent. Cell aggregation was completely inhibited by keeping the cell culture at 4°, whereas strongly aggregation was observed at 37° (Table 4).

U937 cell aggregation induced by M6-1D4 is LFA-1/ICAM-1-dependent pathway

To determine whether LFA-1 (CD11a/CD18) and ICAM-1 (CD54) play a role in CD147 mAb-induced cell aggregation, a mAb inhibition test was performed. As shown in Table 5, MEM-25 (CD11a) and MEM-111 (ICAM-1) mAbs completely blocked M6-1D4 induced cell aggregation. In contrast, MEM-30, another CD11a mAb, and MEM-48 (CD18) mAb had no inhibitory effect on cell aggregation induced by M6-1D4. In the absence of M6-1D4, all of these tested mAbs did not induce cell aggregation (data not shown).

Expression of adhesion molecules on M6-1D4 mAb-treated U937

To determine whether M6-1D4 mAb up-regulates adhesion molecule expression, M6-1D4 mAb-treated U937 cells were stained with PE-labelled HI111(CD11a), ICRF44 (CD11b), B-ly6 (CD11c), 6.7 (CD18), HA58 (CD49d) and HA58

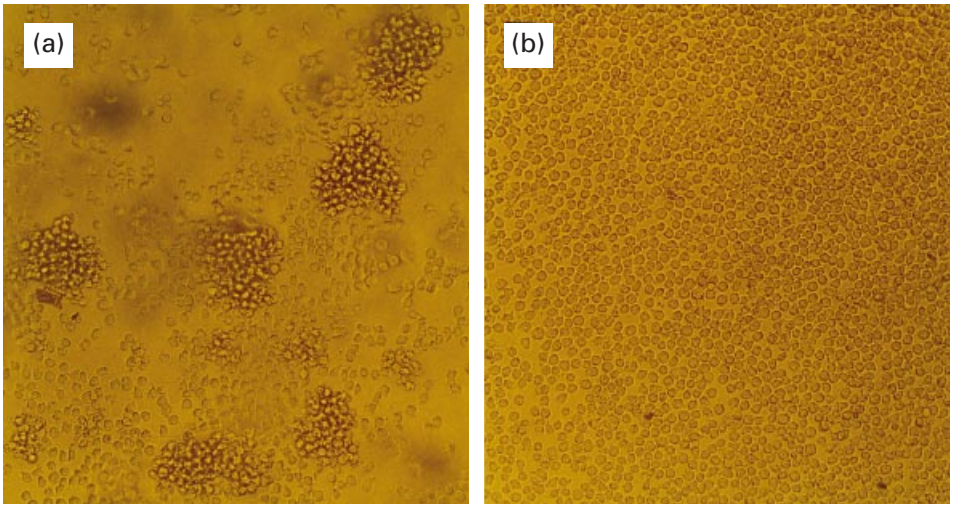

Figure 4. Photomicrographs (×100) of homotypic cell aggregation induced by M6-1D4 mAb. U937 cells were incubated for 24 hr with 10 μg/ml M6-1D4 mAb (a) or MT4 isotype matched control mAb (b).

Table 3. Time kinetics of M6–1D4 mAb in induction of U937 homotypic aggregation

	Incubation time (hr)							
Cells	2	4	8	18	24	48	72	96
U937	–	–	–	2+	4+	4+	4+	4+
K562	–	–	–	–	–	–	–	–

U937 or K562 cells were cultured for 2, 4, 8, 18, 24, 48, 72 and 96 hr in the presence of M6-1D4 mAb (10 μg/ml). Homotypic cell aggregation was determined under an inverted microscope. Degree of cell aggregation was graded as described in Materials and Methods. Results are representative of five separate experiments.

Table 4. Effects of EDTA, sodium azide and temperature on homotypic aggregation of U937 induced by M6-1D4 mAb

Treatment	Concentration	Homotypic aggregation
None		–
M6-1D4		4+
MT4		–
M6-1D4 plus		
EDTA	0·1 mM	4+
	0·5 mM	4+
	1·0 mM	–
Sodium azide	0·0125%	4+
	0·025%	4+
	0·05%	4+
	0·1%	1+
4°*		–

U937 cells were incubated at 37° for 24 hr with M6-1D4 mAb in the presence or absence of various concentrations of EDTA or sodium azide. Homotypic cell aggregation was determined under an inverted microscope. Degree of cell aggregation was graded as described in Materials and Methods.

*U937 cells were incubated at 4° for 24 hr in the presence of M6-1D4 mAb. Results are representative of two separate experiments.

Table 5. Effect of LFA-1 and ICAM-1 mAbs on U937 cell aggregation induced by M6-1D4 mAb

Blocking mAb	CD designated	Degree of cell aggregation
None	–	4+
MEM-25	CD11a	–
MEM-30	CD11a	4+
MEM-48	CD18	4+
MEM-111	CD54	–

U937 cells were incubated at 37° for 24 hr with M6-1D4 mAb in the presence or absence of blocking mAbs (40 μg/ml). Homotypic cell aggregation was determined under an inverted microscope. Degree of cell aggregation was graded as described in Materials and Methods. Results are representative of two separate experiments.

(CD54) mAbs. It was found that binding of M6-1D4 mAb to the CD147 molecule on U937 did not alter the intensity of the expression of all molecules tested (Fig. 5 and data not shown).

DISCUSSION

CD147 molecule is a human leucocyte surface protein which has been recently clustered at the 6th International Workshops on Human Leukocyte Differentiation Antigen.[1] The cellular function of this molecule was investigated and suggests that it is a potential adhesion molecule with an unknown counter-receptor.[1] In this paper, we have demonstrated that CD147 molecule plays an important role in induction of homotypic cell aggregation because incubation of mAbs specific to CD147 molecule with U937 cells results in homotypic cell aggregation. The CD147 mAb-induced cell aggregation is LFA-1/ICAM-1-dependent pathway.

In this study, the CD147 mAbs were generated after immunizing mice with CD147-expressing cell lines and differentially screening hybridoma supernatants by using CD147 expressing transfected COS cells as specific antigen. Six hybridoma clones producing antibodies specific for CD147 were obtained. Immunoprecipitation of biotin labelled U937 and Molt4 cells

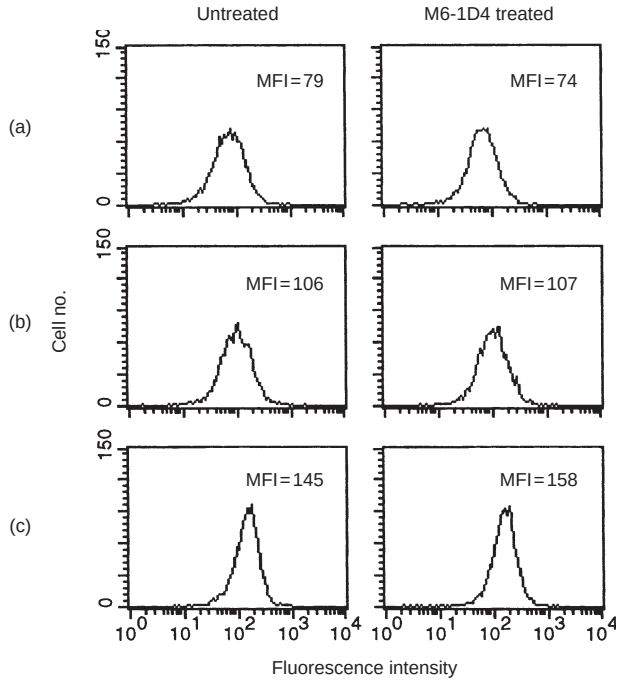

Figure 5. Expression patterns of LFA-1 and ICAM-1 molecules on the surface of U937 treated and untreated with M6-1D4 mAb. U937 cells were cultured in the presence or absence of M6-1D4 mAb for 24 hr. Cells were stained with PE-labelled HI111 (CD11a) (a), PE-labelled 6·7 (CD18) (b) or PE-labelled HA58 (CD54) mAb (c). Mean fluorescence intensity (MFI) are indicated. One representative experiment of two is shown.

reveals that all CD147 mAbs precipitated a broad protein band of ≈45 000–65 000 MW under reducing conditions, which is the same as described in a previous report.[1] However, this molecular weight was higher than that obtained from HSB-2 cells.[10] It is likely that CD147 antigen expressed on HSB-2 cells is somewhat less glycosylated than on other cell populations.[10] In the present immunoprecipitation studies, interestingly, the broad precipitated protein band was composed of multiple protein bands. This result suggests that CD147 is expressed as a large number of structurally different isoforms. These isoforms may result from differential glycosylation.

Cellular expression analysis of CD147 molecules using the generated CD147 mAbs indicated that CD147 protein is broadly expressed on haemopoietic cell lines. Within peripheral blood cells, CD147 molecule expressed on all blood cells and is an lymphocyte activation-associated cell surface protein, as already reported.[1,3,10] The CD147 antigen, however, appears on PHA activated lymphocytes at a later phase compared to CD25[16] or CD71 expression.[16] With red blood cells, only two out of six generated CD147 mAbs showed positive reactivity. The different binding pattern of CD147 mAbs is unlikely because of the effect of the addition of sialic acid; neuraminidase treatment had no effect on the binding of these CD147 mAbs.

Intercellular adhesive events are involved in a wide range of biological processes, including pattern formation and morphogenesis during development, immune responses, leucocyte recirculation, wound repair, tumor growth and metastasis.[17,18] Homotypic cell aggregation is an adhesive phenomenon and is related to intercellular activation. In the present study, we found that CD147-specific mAbs can induce homotypic aggregation of U937 cells. The aggregation began to appear 18 hr and reached maximum after 24 hr in the presence of CD147 mAb for the entire culture period. Cell aggregation induced by CD147 mAb requires a longer period than those induced by other mAbs including CD44,[19] LFA-1,[20,21] ICAM-1[20] or mNI-11 mAb.[22] A lag in homotypic aggregation induced by CD147 mAb may indicate the requirement of RNA or *de novo* protein synthesis. The mechanisms involved in the induction of cell aggregation by CD147 mAb are currently being investigated.

The CD147 mAb-induced homotypic cell aggregation is unlikely because of the cross-linking of antigens; while CD147 mAbs could induce cell aggregation, the isotype-matched controls, MT4 and P-3E10, which are also bound to U937 (data not shown), did not induce cell aggregation. CD147 mAb-induced cell aggregation occurred when cells were cultured at 37°, but never occurred at 4°. Furthermore, we have shown that the cell aggregation induced by CD147 mAb was inhibited by LFA-1 and ICAM-1 mAbs. Moreover, K562 which also strongly express CD147 molecules did not show homotypic cell aggregation in response to CD147 mAbs.

LFA-1 and ICAM-1 are known to be the representative molecules participating in cell aggregation.[18,23–28] Monoclonal antibodies to several leucocyte surface molecules have been reported to induce homotypic aggregation via LFA-1/ICAM-1.[24–26,28,29] In order to investigate whether LFA-1/ICAM-1 molecules are involved in the induction of U937 homotypic aggregation by CD147 mAbs, LFA-1 and ICAM-1 mAbs were used to inhibit cell aggregation. U937 cell aggregation induced by M6-1D4 (CD147) mAb was abrogated by CD11a and ICAM-1 mAbs. These results indicated that the aggregation induced by M6-1D4 mAb is mainly mediated by LFA-1/ICAM-1-dependent pathway. The failure of the MEM-48 (CD18) mAb and MEM-30 (CD11a) mAb in inhibition of cell aggregation is likely that these antibodies recognized the epitopes that were not involved in cell adhesion. MEM-48 is a so-called 'activating antibody' of the LFA-1/ICAM-1 pathway of adhesion.[30] This antibody enhanced LFA-1-mediated T-cell adhesion to ICAM-1 molecule.[30] MEM-30 mAb has been previously shown to be an aggregation-inducing antibody[31] and a poor blocking antibody of phorbol myristate acetate (PMA)-induced cell aggregation.[32]

Increased expression or alteration of cell surface charge by changes in glycoprotein sialylation of adhesion molecules have been reported to initiate cell adhesion.[18,33–35] In this study, increased expression of LFA-1 and ICAM-1 molecules on U937 by CD147 mAbs is unlikely as the density of LFA-1 and ICAM-1 molecules before and after treatment with M6-1D4 was not different. Induction of cell aggregation by M6-1D4 mAb, therefore, may be a result of the fact that the binding of M6-1D4 mAb to CD147 protein converts the adhesion molecules from a low to high avidity state. This is in accordance with a previous paper which described the conversion of LFA-1 molecules by cross-linking the T-cell receptor (TCR) with mAb.[36]

M6-1D4-induced U937 aggregation was inhibited in the presence of EDTA (1 mM) indicated that aggregation is

divalent cation dependent. This result appears to be agree with the previous finding that LFA-1/ICAM-1 adhesion is divalent cation requirement.[37] M6-1D4-induced U937 aggregation also blocked by sodium azide or incubation at 4°, implied that it requires intact cell metabolism.

In summary, in this study, we have demonstrated a functional role of CD147 molecule. Our observations suggest that binding of CD147 mAb, which mimics the natural ligand binding, may generate intracellular signals that activate LFA-1/ICAM-1 intercellular adhesion pathway. This intercellular adhesion event may be involved in immune responses or other biological processes which remain to be determined.

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